Biology of acute myeloid leukemia stem cells

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Differential redox-regulation and mitochondrial dynamics in normal and leukemic hematopoietic stem cells: A potential window for leukemia therapy

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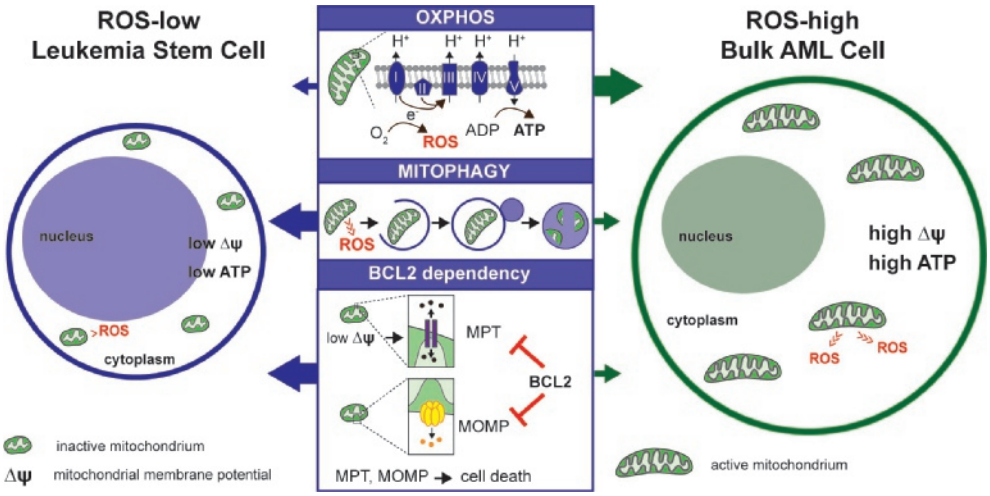
Abstract

The prognosis for many patients with acute myeloid leukemia (AML) is poor, mainly due to disease relapse driven by leukemia stem cells (LSCs). Recent studies have highlighted the unique metabolic properties of LSCs, which might represent opportunities for LSC-selective targeting. LSCs characteristically have low levels of reactive oxygen species (ROS), which apparently result from a combination of low mitochondrial activity and high activity of ROS-removing pathways such as autophagy. Due to this low activity, LSCs are highly dependent on mitochondrial regulatory mechanisms. These include the anti-apoptotic protein BCL2, which also has crucial roles in regulating the mitochondrial membrane potential, and proteins involved in mitophagy.
Here we review the different pathways that impact mitochondrial activity and redox-regulation, and highlight their relevance for the functionality of both HSCs and LSCs. Additionally, novel AML therapy strategies that are based on interference with those pathways, including the promising BCL2 inhibitor Venetoclax, are summarized.



Graphical abstract. *Leukemia stem cells (LSCs) have low levels of ROS and rely on mitochondrial integrity for their survival. In AML cells, ROS levels and stemness potential are inversely correlated, which allows discrimination of ROS-low LSCs and ROS-high bulk leukemic cells. LSCs maintain their characteristic ROS-low state through low levels of mitochondrial oxidative phosphorylation (OXPHOS) and high levels of mitophagy. LSCs are highly dependent on BCL2 mediated mechanisms for maintenance of their remaining mitochondrial activity and for preventing induction of cell death pathways such as mitochondria permeability transition (MPT) and mitochondrial outer membrane permeabilization (MOMP). Interference with BCL2 is a potential therapy strategy for LSC-selective targeting. ATP: adenosine triphosphate.*

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1 Introduction and scope of this review

Current treatment strategies for acute myeloid leukemia (AML) result in an initial reduction of leukemic blasts in the majority of patients. However, a small population of leukemia stem cells (LSCs) persists during therapy and leads to disease relapse. In order to improve therapy success, AML research has focused on studying the molecular mechanisms that underlie LSC properties, and highlighted common as well as distinct features of LSCs compared to normal hematopoietic stem cells (HSCs). The functionality of HSCs is closely connected to their ability to avoid accumulation of oxidative stress and maintain low levels of reactive oxygen species (ROS). With increasing levels of ROS, HSCs capability for long-term repopulation declines, suggesting that low levels of ROS might be an indicator of stem cell functionality.[1,2] Several recent studies have supported the idea that a similar concept applies to LSCs.[3–5] LSCs are a relatively rare fraction of self-renewing malignant cells responsible for disease maintenance.[6] Interestingly, even though a ROS-low state is indicative for both HSC and LSC function, the two cell populations seem to have an altered dependency on pathways regulating ROS production and mitochondrial health. HSCs rely on glycolysis as their main energy source, which results in less oxidative burden compared to mitochondrial oxidative phosphorylation (OXPHOS). Mitochondrial activity and ROS production in HSCs are often induced by either growth factors or cytokines that promote cell differentiation or apoptosis. Although LSCs also have characteristically low levels of OXPHOS,[3,5,7] they depend on this remaining activity for their survival. Consequently, they ensure low ROS levels and high mitochondrial integrity through mechanisms such as autophagy.[4] Moreover, LSCs are highly dependent on pathways that counteract mitochondrial dysfunction and cell death. These LSC-specific characteristics led to the hypothesis that LSCs can be selectively targeted during AML treatment while sparing HSCs.

To address this hypothesis, we begin this review by describing mitochondrial ROS generation and removal, and discuss the relevance of mitochondrial activity for the maintenance and differentiation of stem cells. For both HSCs and LSCs we then summarize the current evidence suggesting that ROS levels are indicative for their functionality. Next, we describe the signaling pathways that have critical roles in regulating redox homeostasis and their potential impact on stem cell maintenance. This includes five modes of signaling – HIF-1α, AMPK, mTOR, FOXO and SIRT – as well as a brief overview of crucial players in DNA damage response pathways. In the final part, we discuss vulnerabilities of LSCs and how interference with autophagy or the anti-apoptotic protein BCL2, which is essential to mitochondrial health, could offer a strategy for targeting these proteins as part of AML treatment.

2 ROS generation and its relevance for stem cell maintenance and differentiation

ROS is a collective term for oxygen-containing molecules that are more reactive than molecular oxygen (O_2), and mainly include hydroxyl radicals

(OH·), hydrogen peroxide (H_2O_2) and superoxide anion radicals ($O_2\cdot^-$). Due their chemical characteristics, ROS can easily react with DNA or RNA bases, fatty acids in lipids or amino acids in proteins.[8] For the majority of mammalian cells, mitochondrial energy production is the major source of ROS.[9] During ATP production by mitochondrial oxidative phosphorylation (OXPHOS), substrates are oxidized by a series of enzyme complexes (complex I-IV) located at the inner mitochondrial membrane. Reactions of this electron transport chain (ETC) lead to release of ROS. Besides ROS production by mitochondria, these molecules are also produced in enzymatic reactions by the family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) and other oxidases involved in inflammatory reactions and other processes. In this review we focus on the role of mitochondrial ROS.

The total amount of steady-state cellular ROS differs between cell types. In general, the primitive stem cell fraction has a lower ROS content during steady-state, while higher ROS levels are found in more differentiated cells. This difference mainly results from metabolic properties that are closely linked to the cellular function.[10] The low ROS content in the stem cell fraction might be protective for DNA damaging effects[11,12] and primitive stem cells use various strategies to avoid ROS generation. For instance, stem cells from different origins have been found to reside in defined anatomical compartments with low oxygen tension, which are called stem cell niches.[13] These hypoxic conditions induce altered metabolic features, including characteristically low mitochondrial activity.[14] Undifferentiated ESC contain small, immature mitochondria, but during differentiation cells acquire a more elongated, mature mitochondrial phenotype, accompanied by higher copy numbers of mitochondrial DNA and elevated ATP production required for cell growth and proliferation.[15] Similarly, HSCs were shown to have relatively low mitochondrial activity and low membrane potential, a feature that was shown to be essential for their stemness potential.[16–21] Quiescent HSCs rely primarily on anaerobic glycolysis for their energy generation.[22] Although this is less efficient than OXPHOS, it also restricts the burden of ROS-mediated oxidative stress (**Figure 1**). However, upregulation of mitochondrial activity in concert with increased ROS levels is crucial for HSC differentiation, since disruption of OXPHOS was shown to impair this process.[23,24]

ROS can drive cell proliferation and differentiation mechanistically via redox modification of proteins critically involved in these processes.[8] In addition, ROS modulate redox-sensitive factors that regulate ROS production itself, such as forkhead homeobox type O family (FOXOs), ataxia telangiectasia mutated (ATM) or Sirtuins (SIRTs). ROS also modulate molecules that have important functions in stem cell maintenance, differentiation or stress response, such as hypoxia-inducible factor 1α (HIF-1α), p38 and p53. ROS-mediated activation of the p38-MAPK (mitogen-activated protein kinase) pathway was shown to have a crucial role in limiting the lifespan and functionality of HSCs.[25–28] P38 phosphorylation – and thus activation – promotes inhibitory programs such as cell-cycle arrest and apoptosis, or can activate purine metabolism, resulting in increased HSC cycling.[29] Furthermore,

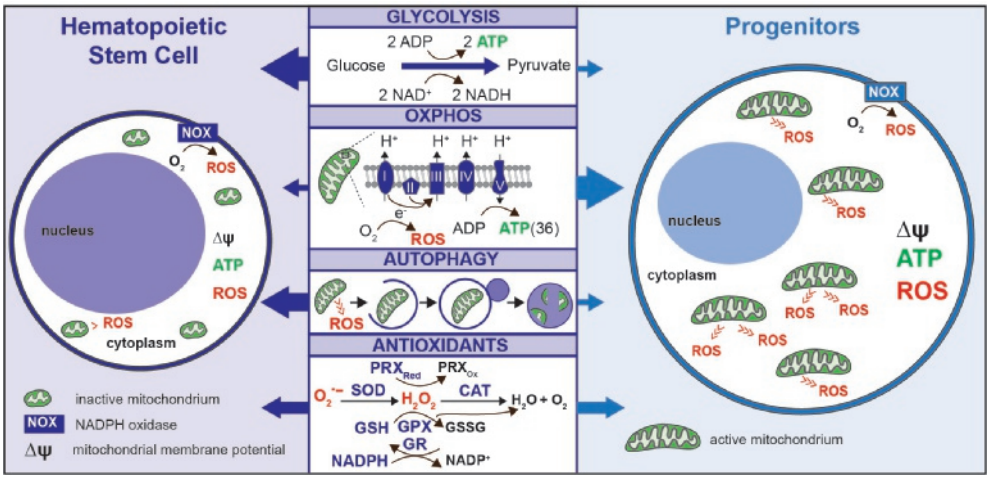

Figure 1. Steady-state ROS levels vary in the different types of hematopoietic cells. Left panel: *Hematopoietic stem cells (HSCs) have characteristically low mitochondrial activity and low levels of reactive oxygen species (ROS), which are maintained by using glycolysis as their main energy source, removing stressed (and ROS producing) mitochondria via autophagy and neutralizing ROS via reactions of anti-oxidative enzymes. HSCs produce less ATP and have smaller mitochondria compared to progenitors.* ***Right panel:*** *More differentiated progenitors characteristically have larger mitochondria, higher levels of mitochondrial activity and oxidative phosphorylation (OXPHOS), increased ATP production and lower levels of autophagy. They also make use of anti-oxidative enzymes to avoid oxidative stress. PRX: peroxiredoxin; SOD: superoxide dismutase; CAT: catalase; GSH: glutathione; GPX: glutathione peroxidase; GR: glutathione reductase; GSSG: glutathione disulfide; NADP: nicotinamide adenine dinucleotide phosphate.*

ROS-induced p53 was found to be important for controlling HSC survival.[30]

3 ROS-regulating mechanisms maintain HSC functionality

To counteract ROS production and prevent oxidative stress, HSCs have numerous ROS-scavenging strategies. These include low-molecular-weight reducing peptides (e.g. glutathione, thioredoxin, NADPH), peroxiredoxins and antioxidant enzymes (e.g. catalase, superoxide dismutase (SOD), glutathione peroxidase). They also have other ROS regulating mechanisms, such as mitophagy, that control mitochondrial quantity and quality (**Figure 1**), or the recently discovered process of CoAlation.[31,32] Additionally, interactions of cells with their microenvironment can impact their ROS levels.[33,34] In steady-state conditions, higher levels of antioxidant enzymes have not been found in HSCs than in more committed progenitors,[35] indicating that the higher availability of antioxidants does not explain the lower ROS levels in HSCs. However, transcription factors regulating the expression of anti-oxidative molecules are essential for HSC functioning.[26,36–38] This suggests that stem cells could be more dependent on anti-oxidative molecules than more committed progenitors. This postulation is also supported by a recent study showing a correlation between the magnitude of ROS-mediated effects and cell size: in smaller cells ROS interact more easily with membranes.[39] Stem cells

are characteristically smaller in size than their progeny[40] and could thus rely more on ROS-scavenging mechanisms. In line with this possibility, HSCs were shown to depend on ROS-regulating pathways such as autophagy, in which cells break down dysfunctional organelles by lysosomal degradation.[41]

Autophagy results in the clearance of ROS-producing mitochondria – a process known as mitophagy – which is especially important for HSC functioning.[42,43] Autophagy is regulated by a number of autophagy-related genes (Atg); *Atg5* and *Atg7* knock-out mice show an increased number of mitochondria and ROS levels in hematopoietic stem and progenitor cells in conjunction with an impaired functionality.[44,45] Similarly, low autophagy levels in aged HSCs are accompanied by high ROS levels and reduced functionality, whereas aged HSCs with high autophagy levels perform comparably to their younger counterparts.[46] Efficient mitophagy is also closely linked to the dynamic mitochondrial fusion/fission process in which damaged mitochondria become segregated by fission (division) and healthy material is exchanged between mitochondria via fusion. Altered expression of fission-proteins DRP1 and FIS1 can impact stem cell maintenance.[4,47]

4 Excessive mitochondrial ROS production triggers cell death pathways

Although physiological ROS levels are important for intracellular signaling, excessive ROS can induce cell death by triggering apoptotic pathways. Because they are the primary cellular location of ROS production, mitochondria are particularly vulnerable to ROS-induced damage. ROS can damage mitochondrial DNA, membrane lipids and proteins (reviewed in [48]), which can result in mitochondrial genomic instability and respiratory dysfunction. ROS can also oxidize and damage components in the inner and outer mitochondrial membrane, resulting in a dysfunctional respiratory chain, a drop in mitochondrial membrane potential and eventually triggering mitochondrial permeability transition (MPT) and cell death.[49] Moreover, accumulation of ROS can induce structural changes in the mitochondrial outer membrane (MOM) and trigger MOM permeabilization (MOMP) mediated by BCL2 family proteins. An important effector of ROS-induced cell death is the pro-apoptotic protein p53. Whereas mild oxidative stress leads to expression of p53-targets with antioxidant function, high ROS levels trigger p53-mediated cell death.[50] Hyper-activation of p53 was shown to deplete HSCs,[30] whereas drug-induced mitochondrial dysfunction resulted in LSC-specific apoptosis in leukemia cells, accompanied by increased ROS levels and p53-activation.[51] Furthermore, inhibition of autophagy – and thereby mitochondrial turnover – was shown to target p53-wildtype leukemias, while being ineffective on cells carrying p53 mutations.[52] In line with these observations, several recent studies have shown the importance of mitochondrial health for the survival and function of both HSCs[1,42,46,53–58] and LSCs.[4,59–64]

Mitochondrial ROS production also plays a central role in a different type of programmed cell death induced by oxidative stress, known as ferroptosis,[65] which is the process of regulated

necrosis induced by iron-mediated lipid peroxidation. In brief, if ROS interact with lipids and remove their free electrons, "lipid ROS" are formed. If lipid ROS accumulate, ferroptosis-mediated cell death is induced.

5 ROS levels in normal hematopoietic stem cells (HSCs)

It was initially assumed that HSCs have a lower number of mitochondria compared to more differentiated progenitors, which could contribute to the low rates of oxidative metabolism observed in HSCs.[24,57,66–68] However, more recent studies have challenged this idea by showing that the lower values of mitochondrial mass observed when using dye-based methods are the result of an altered expression of efflux pumps in HSCs and not of a lower number of mitochondria. Instead, these researchers proposed that HSC contain a relatively high number of less active mitochondria.[17,69] Notably, the accumulation of mitochondrial mutations in HSCs did not impair the maintenance of HSCs per se, but rather their capability to give rise to functional progenitors. This finding supports the notion that oxidative metabolism is less important for HSCs themselves, but is crucial for their repopulation capacity.[17]

Studies that compared ROS levels between stem cells and progenitor cells found that HSCs and megakaryocyte-erythroid progenitors (MEPs) have the lowest ROS levels, whereas granulocyte-macrophage progenitors (GMPs) have the highest ROS levels.[70,71] This observation is possibly in line with the results of several recent studies suggesting that the megakaryocytic lineage directly evolves from HSCs.[72–77] Growth factors that promote myeloid proliferation and differentiation, as well as cytokines that are released upon tissue damage,[78] often induce mitochondrial biogenesis and ROS production. This correlates with HSC differentiation or exhaustion.[1,2] However, there is still a dynamic range in levels of ROS within the phenotypically defined steady-state HSC population that is not exposed to any additional stressors, which is indicative of their functionality. In 2007, Jang and Sharkis separated viable murine Lin^-CD45^+ bone marrow cells into a ROS-low and ROS-high fraction based on their signal intensity for the fluorescent ROS-dye DCFDA (2',7'-dichlorofluorescin diacetate).[79] ROS-low HSCs were shown to have higher self-renewal potential, whereas serial transplantation assays with ROS-high HSCs exhausted faster than their ROS-low counterparts due to increased activation of the p38-MAPK. More recently it was shown that TNF secretion by bone marrow cells and subsequent elevation of ROS can fluctuate between different times of the day based on influences of light and darkness. This determines whether HSCs self-renew (when ROS levels are reduced) or differentiate (when ROS levels are higher).[80] Besides ROS-dependent variability in HSC function during steady state, numerous mouse gene knock-out studies reported that a stress-induced increase in ROS results in impaired HSC function or exhaustion.[26,36,81,82] Furthermore, it was also shown that exposure of HSCs to ambient oxygen during the procedure of stem cell transplantation can lead to extra-physiologic oxygen shock/stress response (EPHOSS), resulting in ROS accumulation and

decreased repopulation potential upon transplantation.[49,83] In line with this finding, long-term engraftment of human HSCs in murine models could be enhanced by overexpression of catalase (a ROS-detoxifying agent)[84] or pre-treatment with valproic acid, which enhanced glycolytic potential and decreased mitochondrial activity, thereby counteracting ROS accumulation.[85] In summary, these studies indicate that HSCs are best maintained under ROS-low conditions. During steady state, these conditions result from their glycolytic metabolism and location in the hypoxic bone marrow niche.

6 ROS levels in Leukemia stem cells (LSCs)

According to the prevailing LSC model, a rare population of malignant cells with properties similar to normal HSCs – such as self-renewal and quiescence – is capable of maintaining the disease. (reviewed in [6]). Moreover, LSCs can initiate leukemia when transplanted into immunodeficient mice,[86] and are therefore alternatively called leukemia initiating cells (LICs). LSCs are functionally defined, do not have a uniform phenotype[87,88] and exhibit great variability in molecular defects.[89] However, despite their heterogeneity, several recent studies have suggested that LSCs share metabolic features that are distinct from the total leukemia cell population and characteristically include low mitochondrial activity and low levels of cellular ROS.[3,4,90,91]

Recently, Hao et al proposed that LSCs are more glycolytic compared to bulk AMLs cells,[90] which would reflect the similar relation that HSCs have compared to their more differentiated progenitors. The investigators used a metabolic sensor called SoNar (sensor for NAD(H) redox) that has different fluorescent properties depending on whether it binds to NADH or NAD+, and thus reflects the cytosolic NADH/NAD+ ratio of a cell. Glycolysis describes the conversion of glucose to pyruvate and leads to production of NADH, which may be further oxidized to NAD+ by mitochondrial reactions or by the enzyme lactate dehydrogenase during lactate production. Hence, high levels of glycolysis accompany NADH accumulation, and SoNar-high cells are assumed to have higher glycolytic activity. SoNar-high cells were found to be highly enriched for LSCs, in conjunction with low levels of mitochondrial membrane potential and high expression of enzymes that block the entry of substrates in the TCA cycle such as the pyruvate dehydrogenase PDK2.

These findings are in line with other recent studies showing that AML LSCs have low levels of oxidative metabolism and reside in the "ROS-low" fraction of the AML mononuclear cell population.[3,4,91] Additionally, our group recently demonstrated that even $CD34^+$ selected stem- and progenitor AML cells have a relatively broad range of ROS levels and that the $CD34^+$/ROS-low fraction is enriched for phenotypic $CD34^+CD38^-$ LSCs (unpublished results). Low levels of ATP were observed in both SoNar-high and ROS-low cells, but these cells also had opposing characteristics. In another study, ROS-low leukemia cells were described as having rather low levels of glycolysis and were enriched for quiescent cells,[3] whereas SoNar-high cells were described as non-quiescent.[90] However, mice transplantation studies

have indicated that both groups of cells are enriched for LSCs.[3,90]

In summary, the above studies suggest that LSCs tend to have low levels of oxidative metabolism and ROS, which coincides with other studies demonstrating that overexpression of glycolysis genes such as *PDK2* and *PDK3* is associated with poor prognosis in AML.[92] Although these observations could lead to the assumption that LSCs are less dependent on oxidative metabolisms for their survival, the opposite seems to be the case. Multiple studies have demonstrated that functional mitochondria biology and OXPHOS are crucial for the survival and maintenance of leukemia cells[3,93–95] Inhibition of mitochondrial translation by Tigecycline resulted in decreased expression of the mitochondrial complex IV subunits COX-1 and COX-2 and impaired survival of AML LSCs.[96] Similarly, Tigecycline-treatment efficiently targeted primitive $CD34^+$ CML LSCs, whereas it did not affect the colony-forming-capacity of normal $CD34^+$ cells.[59] When mitochondrial activity is blocked, LSCs may also have decreased ability to switch to glycolysis as their main energy source.[3] Furthermore, treatment of primary AML blasts with a small molecule inhibitor of complex I of the mitochondrial electron transport chain (ICAS-010759) induced apoptosis, while treatment of normal bone marrow cells did not affect viability.[98] Additionally, LSC survival was impaired not only by inhibition of mitochondria themselves, but also by pathways that generate substrates for the mitochondrial TCA cycle, including inhibition of fatty acid oxidation,[93] glutaminolysis[97] and amino acid metabolism.[5]

7 ROS-regulating pathways and their importance for stem cell functionality

As summarized above, low levels of ROS are indicative for both HSCs and LSCs. In line with this notion, pathways that contribute to a cellular ROS-low state are often found to function as guardians of stemness. These pathways are outlined in this section. Key players for controlling cellular ROS levels are involved not only in the regulation of metabolic pathways, but also in important stress response mechanisms such as DNA repair or autophagy. Furthermore, effective functioning of pathways that mediate mitochondrial health is essential for both HSC and LSC functionality (**Figure 2**).

7.1 HIF signaling

The transcription factors hypoxia-inducible factor (HIF) 1α and 2α have key roles in translating changes of the cellular environment and nutrient availability into a transcriptional response. HIF-1α and HIF-2α both form heterodimers composed of α constitutively-expressed β-subunit and an α-subunit that is degraded by an oxygen-depended hydrolase. Consequently, it is present only under hypoxic conditions.[99] HIF-1α was shown to be highly expressed in HSCs with long-term repopulating capacity[16] and has been described as a master regulator of metabolic pathways that contribute to a ROS-low environment.[100–102] In brief, HIF-1α stimulates glycolysis by inducing expression of glycolytic enzymes (e.g. HK1, LDHA) and glucose transporters (e.g. GLUT1), and inhibits mitochondrial OXPHOS by inducing expression of enzymes (e.g. PDK2, PDK4) that block

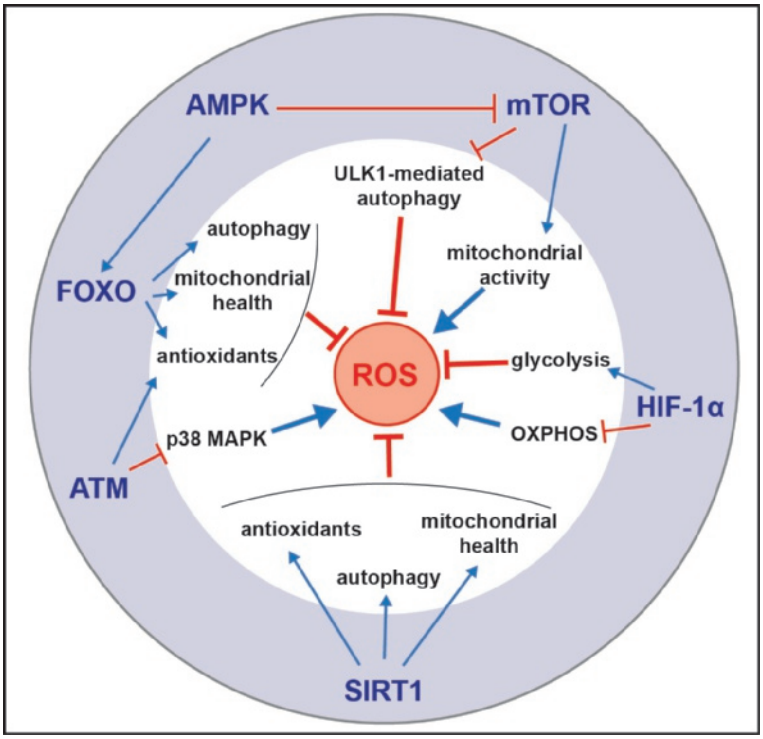

Figure 2. A crosstalk of signaling pathways determines the final ROS concentration and impacts stemness. *Various pathways influence ROS levels by regulating mitochondrial activity, glycolysis, autophagy, expression of antioxidative enzymes or stress-responsive signaling cascades. AMPK: AMP-activated protein kinase; mTOR: mammalian target of rapamycin; FOXO: forkhead box class O family; ATM: ataxia telangiectasia mutated; hypoxia-inducible factor 1α (HIF-1α); SIRT1: sirtuin 1; p38 MAPK: p38 mitogen activated protein kinase.*

entry of substrates into the TCA cycle.

Although no consensus has been reached on whether HIF expression is necessary for full functionality of HSCs, multiple studies have indicated that HIF plays a role in HSC function: Conditional deletion of *Hif-1α* itself[103] or its upstream regulator *Meis1*[16] was shown to impair HSC quiescence. *Meis1*-/- HSCs showed increased ROS production, loss of HSC maintenance under stress conditions and increased HSC apoptosis, which is a phenotype that could be entirely rescued by treatment with NAC.[104,105] Furthermore, depletion of the HIF-1α target gene *Ldha*, a subunit of the enzyme lactate dehydrogenase (LDH) that regulates the last step of anaerobic glycolysis, increased ROS levels in mouse bone marrow and impaired HSC maintenance.[106] Similarly, deletion of the HIF-1α target genes *Pdk2* and *Pdk4* in murine HSCs impaired HSC transplantation capacity and resulted in increased levels of ROS and expression of senescence markers.[24] Also, knockdown of the HIF-1-inhibitor CITED2 (CBP/p300-interacting-transactivator-with-an-ED-rich-tail 2) was shown to affect

HSC maintenance[107–109] and result in impaired glycolysis with elevated cellular ROS levels.[110] This suggests that tight regulation of HIF levels is crucial for maintaining HSC metabolism.

However, other recent studies reported that individual or combined deletion of *HIF-1* and *HIF-2* in HSCs does not affect their self-renewal and repopulation capacity, also in the context of serial transplantations or 5-fluorouracil treatment.[111–113] This disparity in results could be related to different experimental designs: Whereas *Hif-1* deletion in both murine HSCs and bone marrow environment affected HSC function,[103] *Hif-1α* deletion only in the hematopoietic cells did not.[113] In line with this explanation, a recent study concluded that stable expression of HIF proteins is less relevant for stem cell maintenance itself, but is instead important for effective mobilization of HSPCs from the bone marrow niche to the peripheral blood.[114] Moreover, other studies have shown that hypoxic conditions can also influence ROS levels and that the ROS-signaling network is in a certain manner independent of HIF.[115,116] Therefore, HIF-mediated ROS regulation might not be fundamental for HSC functionality.

Similarly to HSCs, the role of HIF signaling for LSCs is also not yet fully understood. A recent study by Raffel et al. indicated that enhanced activity of enzymes that target HIF-1*α* for degradation can decrease AML stem cell maintenance,[117] thus supporting a role for HIF-1 in LSC function. Similarly, decreased expression of HIF-2*α* and CITED2 induced by anti-diabetic drug treatment was suggested as being involved in CML-LSC elimination.[118] In contrast, LSCs lacking both HIF-1α and HIF-2α are still capable of promoting AML development[112] and *HIF-1α*-deleted leukemia cells showed an even faster disease progression after chemotherapy.[119] Consequently, the role of HIFs in LSCs and leukemia pathogenesis remains unclear.

7.2 FOXO signaling

Transcription factors of the forkhead box class O (FOXO) family are important to the oxidative defense machinery and stimulate the expression of genes coding for antioxidant proteins such as SOD, catalase and sestrin (reviewed in [120]). FOXO proteins (including FOXO1a, FOXO3a, FOXO4 and FOXO6 in humans) are normally present in an active state in the cell nucleus, but are exported to the cytoplasm upon phosphorylation, frequently by the AKT kinase downstream of the PI3K-signaling pathway. However, other factors such as the NAD+ dependent deacetylase SIRT1 can also impact the subcellular localization of FOXOs.[121,122] The family member FOXO3a was shown to be essential for HSC maintenance, since *Foxo3a*-/- HSCs failed to support long-term reconstitution of hematopoiesis and were accompanied by increased ROS levels, activated p38 MAPK signaling and defective DNA damage repair.[26,123] FOXOs are also involved in glucose metabolism by regulating phosphenol-pyruvate carboxykinase (PEPCK) and Glucose-6-phosphatase (G6Pase), two enzymes involved in gluconeogenesis.[124] Intriguingly, Rimmelé et al. demonstrated that elevated ROS levels observed in *Foxo3*-/- HSCs were not the result of a shift from glycolytic towards mitochondrial metabolism, nor did they have a causative role in impaired HSC

functionality.[18] Besides high ROS levels, *Foxo3*-/- HSCs had increased glycolysis levels, decreased OXPHOS and were associated with abnormalities in mitochondrial membrane potential and mass, indicating an important role of FOXOs for mitochondrial metabolism.

A previous study showed that *in vivo* treatment of *Foxo1/2/3*- triple knockout mice with NAC could rescue the impaired repopulation capacity of HSCs in mice transplantation studies when treatment was continued for 5 weeks.[36] However, Rimmelé et al. showed that a NAC-mediated rescue of dysfunctional *Foxo3a*-/- HSCs in repopulation studies was only short-term and was lost at 8 weeks after transplant, despite sustained lowering of ROS levels.[18] This indicates that elevated ROS levels in FOXO-deficient HSCs are not the main cause for their dysfunction; instead, this may reflect an abnormal mitochondrial function. This notion is supported by a study showing that FOXOs promote mitochondrial integrity by stimulating autophagy. FOXOs induce upregulation of the enzyme glutamine synthase, which consequently stimulates glutamine production.[125] High glutamine levels block the mTOR-signaling pathway, which is a negative regulator of autophagy.

7.3 AMPK signaling pathway

The AMP-activated protein kinase (AMPK) is a master regulator of ROS production and elimination. Activated AMPK signaling inhibits the ROS-generating mammalian target of rapamycin (mTOR)- pathway and activates FOXO signaling, which promotes a ROS detoxifying cascade and stimulates ULK1-mediated autophagy that helps to remove damaged organelles. As its name suggests, AMPK is activated by the binding of AMP, which thus takes place in conditions where the AMP/ATP ratio is high. Upstream activators include the kinases LKB1 and CaMMK. Activation of AMPK by the diabetes drug metformin was shown to increase *ex vivo* maintenance of murine HSCs.[126] However, deletion of AMPK in murine HSC only moderately effected HSC function,[127–129] potentially highlighting the role of AMPK in HSC maintenance under certain stress conditions.

In contrast to its function in HSCs, AMPK seems to be more relevant for the survival of LSCs by protecting them from metabolic stress. AMPK deletion in AML LSCs was shown to result in increased ROS levels and DNA damage, as well as reduced glucose flux due to impaired glucose transporter expression, which significantly delayed leukemogenesis.[130] Pei et al. showed that AMPK is intrinsically activated in LSCs and regulates LSC mitochondrial dynamics, thereby conferring LSCs with increased resistance to mitochondrial stress.[4] Additionally, increased AMPK activation was shown to mediate resistance of AML cells to certain epigenetic agents by stimulating ULK1-mediated autophagy,[131] which potentially protects cells from accumulation of ROS and oxidative damage and highlights elevated AMPK signaling as an important survival strategy of LSCs.

7.4 mTOR signaling

AMPK indirectly inhibits the mTOR pathway by phosphorylating – and thereby activating – tuberous sclerosis complex 2 (TSC2), which is part of the TSC1-TSC2 complex that inhibits

mTOR1. The mTOR signaling pathway is activated when the availability of nutrients and ATP is high; this pathway promotes anabolic processes like protein synthesis and mitochondrial activity, but inhibits autophagy. In opposition to AMPK, the pro-proliferative AKT pathway activates mTOR (reviewed in [132]). Prevention of mTOR hyper-activation is essential for preservation of the HSC self-renewal capacity.[79] *Tsc1* deletion in HSCs was shown to dramatically increase ROS production and to drive quiescent HSCs into rapid cycling,[133] whereas treatment of cultured mouse bone marrow cells with the mTOR-inhibitor rapamycin preserved HSPCs.[134] Furthermore, expression of miRNAs that target the mTOR pathway was shown to be critical for preserving long-term repopulating HSCs, while their loss resulted in enhanced mitochondrial biogenesis, metabolic activity and ROS production in HSCs.[56]

mTOR promotes mitochondrial activity in multiple ways. First, it stimulates translation of mitochondria-related genes by phosphorylating eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BPs), which leads to their dissociation from eIF4E and assembly of the translation initiation complex.[135] Hypo-phosphorylation of 4E-BP is characteristic for HSCs and is important for their functionality.[136] Second, mTOR stimulates activity of the transcriptional regulator PPARγ coactivator-1α (PGC-1α), a master regulator of mitochondrial metabolism and activator of mitochondrial fatty acid oxidation genes.[1,137] Of note, PGC-1α also stimulates gluconeogenesis and is induced by stemness-associated genes such as CITED2, highlighting that different pathways can influence the impact of PGC-1α signaling on ROS levels in an opposite way.[138,139] Finally, mTOR1 phosphorylates and inactivates the pro-autophagic kinase ULK1[140] and thereby inhibits autophagy, resulting in the accumulation of mitochondria.[46]

7.5 DNA damage response pathways

Mouse models in which genes are deleted that participate in DNA damage response frequently show a similar phenotype of dysfunctional HSCs (reviewed in [141]). For the genes *ATM* (ataxia telangiectasia mutated) and *MLL5* (Mixed-Lineage-Leukemia-5), the phenotype of dysfunctional HSCs lacking their expression has been proposed to be causally connected to accumulation of ROS.[81,142] The ATM protein kinase is activated by DNA double strand breaks and modulates the activity of various targets to maintain genomic stability by initiating an effective DNA damage response.[143] These targets include numerous antioxidant enzymes that prevent increased ROS levels and oxidative stress.[144] Ito et al. showed that increased ROS levels in *Atm*-/- HSCs led to an impaired self-renewal capacity, which could be restored by treatment with anti-oxidative agents.[81] Mechanistically, high ROS levels in *Atm*-/- HSCs have been shown to activate the p38 MAPK pathway and upregulate p16INK4a, which is associated with induction of HSC senescence.[145] A comparable observation regarding HSC dysfunctionality was made in *Mll5*-/- mice, which showed accumulation of DNA damage, ROS-mediated upregulation of p16INK4a and reversal of the phenotype when treated with the antioxidant NAC.[142] In summary, defective DNA damage response can

result in increased ROS production or impaired ROS elimination, showing that DNA damage response pathways play a crucial role in redox homeostasis and HSC maintenance.

7.6 SIRT1 signaling

Sirtuins are a family of NAD+ dependent lysine deacetylases with seven members in mammals (SIRT1-7), of which SIRT3, SIRT4 and SIRT5 are localized exclusively within the mitochondria. The activity of sirtuins can be influenced by oxidative stress at multiple levels: ROS can induce posttranslational modification, change SIRT expression or protein-protein interactions, or affect cellular NAD levels.[146] The enzymes can be viewed as metabolic sensors in a cell; they play an important role in mediating cellular stress responses or inducing metabolic changes by regulating the activity of targets such as p53, FOXOs, E2F1 or PGC-1*α* (reviewed in [147]). Increased activation of SIRTs eventually results in elevated expression of anti-oxidants such as SOD2 and catalase, as well as stimulation of autophagy, thereby reducing cellular ROS. Additionally, SIRTs promote mitochondrial biogenesis and increased turnover of mitochondria, thereby stimulating removal of damaged mitochondria that otherwise would produce excessive ROS.

Several SIRT family members appear to play a crucial role in HSC functioning under stress conditions. For example, deletion of *SIRT1* in HSCs has been shown to impair HSC homeostasis and to induce an aging-like phenotype by altered regulation of FOXO3.[148] Deletion of *SIRT3* was shown not only to be indispensable for maintenance of young HSCs, but also to be essential under stress conditions or during ageing.[149] SIRT6 was shown to regulate HSC function by suppressing Wnt target genes, and deletion of *SIRT6* led to impaired HSC self-renewal ability.[150] SIRT1 was found to be consistently overexpressed in AML,[151] and was associated with increased leukemia cell survival, proliferation and drug resistance,[152,153] suggesting that SIRT1 also promotes maintenances of LSCs. Nevertheless, SIRT1 deficiency was shown to enhance – and not suppress – the maintenance of LSCs from MDS (myelodysplastic syndrome) patients by leading to decreased activity of the tumor suppressor Tet methylcytosine dioxygenase 2 (TET2),[154] indicating that SIRTs have distinct, context-dependent functions in HSC/LSC maintenance. Furthermore, SIRT2 has been shown to be involved in metabolic reprogramming of leukemia cells by stimulating the pentose phosphate pathway,[155] highlighting an important role of SIRTs in both leukemia proliferation and maintenance via multiple routes.

8 Strategies for targeting Leukemia Stem Cells

HSCs and LSCs are both characterized by low ROS levels and impairment of various ROS-regulating pathways can influence both HSC and LSC function. However, the two populations appear to depend on different pathways to maintain their characteristic redox state or to prevent them from going into apoptosis. As mentioned earlier, LSCs rely on mitochondrial integrity and metabolism for their survival, and mitochondrial ATP generation is crucial for leukemia progression. However, due to the altered metabolic properties of

LSCs and their relatively low levels of mitochondrial membrane potential,[3,90,156] they are potentially more prone to undergo mitochondrial permeability transition (MPT) or apoptosis.[157] Therefore, it is likely that LSCs have an increased dependence on factors that counteract programmed cell death or protect them from stress-induced impairment (oxidative or otherwise) of mitochondrial function, which could provide a window for leukemia therapy. This concept is supported by multiple recent studies that reported increased dependency of LSCs on the anti-apoptotic BCL2 or on stress-response mechanisms such as autophagy (**Figure 3**), which are be described below in more detail.

8.1 Compromising mitochondrial functionality of ROS-low LSCs by BCL2 inhibition

BCL2 can be seen as a master regulator of mitochondrial physiology and cellular stress response. In its canonical role, BCL2 functions as an anti-apoptotic protein by preventing mitochondrial outer membrane permeabilization (MOMP) via its interaction with the pro-apoptotic proteins BAX and BAD. However, BCL2 has also additional functions that influence mitochondrial activity and the cellular redox state by regulating both pro-oxidative and anti-oxidative processes.[158,159] BCL2 was shown to interact with the COX Va-subunit of complex IV that is part of the electron transport chain (ETC), thereby potentially facilitating the transport of this subunit to the mitochondria and increasing mitochondrial activity.[160] Furthermore, BCL2 was found to promote the transport of the anti-oxidant glutathione to mitochondria via direct interaction.[161] In line with this, high levels of BCL2 were shown to provide increased resistance against a decline in membrane potential and MPT induction by maintaining reduced pyridine nucleotides.[162] As early as 1993, BCL2 was shown to function as an anti-oxidant,[163,164] and later studies demonstrated that BCL2 overexpression shifts the redox-state of a cell to a more reduced state.[165,166] Consequently, BCL2 also influences activation of redox-sensitive transcription factors such as NF-κB, p53, and AP-1, which have a crucial role in stress response. In summary, BCL2 preserves mitochondrial stability by preventing a drop of the mitochondrial membrane potential by both stimulating mitochondrial activity and functioning as an anti-oxidant that counteracts the increased ROS production (**Figure 3A**).

In 1994, Campos et al. showed that BCL2 inhibition affects the survival of leukemic stem cells and progenitor cells and improves the efficacy of chemotherapeutic drugs.[167] More recently, several studies indicated that BCL2 inhibition efficiently targets LSCs.[3,5,91,168–170] Lagadinou et al. reported that LSCs – which they defined as the fraction of total mononuclear AML cells with the lowest ROS levels – are especially sensitive to BCL2 inhibition due to their higher BCL2 expression compared to their non-LSC counterparts.[3] Mechanistically, BCL2 inhibition was found to impair OXPHOS, which is crucial for LSC survival.[3,7] Additionally, results from our group indicate that ROS-low $CD34^+$ AML cells are more sensitive to the BCL2 inhibitor Venetoclax[171] compared to ROS-

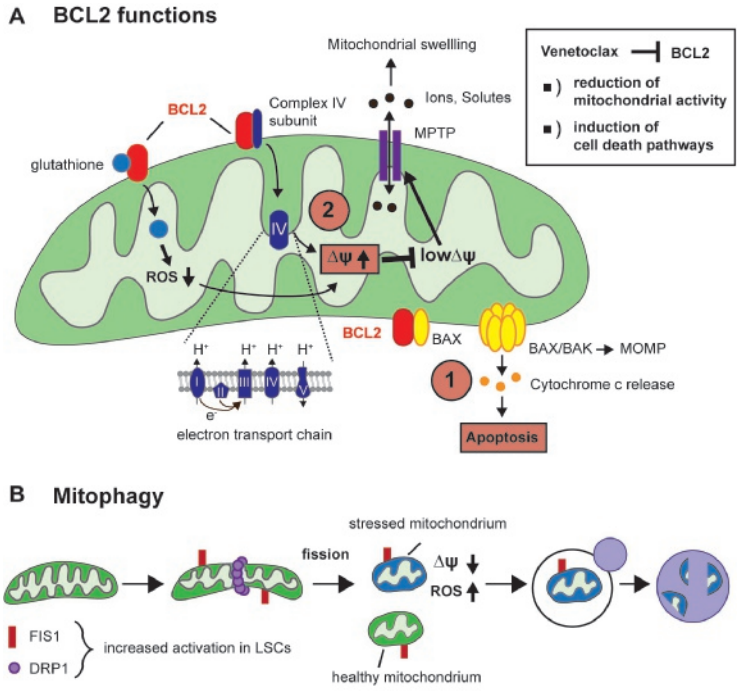

Figure 3. LSCs have increased dependence on BCL2 mediated mitochondrial regulation and mitophagy. ***(A)*** *BCL2 regulates mitochondrial function mainly via 2 routes. 1:The canonical function of BCL2 inhibits apoptosis by preventing oligomerization of the pro-apoptotic proteins BAX and BAK, which otherwise can induce mitochondrial outer membrane permeabilization (MOMP). 2: BCL2 facilitates the import of the anti-oxidant glutathione and complex IV subunits into the mitochondria.This positively affects the mitochondrial membrane potential (ΔΨ) and counteracts its drop to prevent induction of the mitochondrial permeability transition pore (MPTP).* ***(B)*** *The processes of fission and mitophagy are tightly connected. Division of mitochondria by fission involves the GTPase DRP1 and the receptor FIS1, and enables separation of dysfunctional mitochondrial parts from healthy ones. Dysfunctional parts are further degraded by mitophagy. Interference with either BCL2 functioning or mitophagy was shown to target LSCs.*

high CD34$^+$ AML cells despite similar BCL2 expression levels (unpublished results), highlighting their increased BCL2 dependency. Interestingly, BCL2 inhibition has been shown to impair the viability of leukemic cells with different backgrounds with regard to karyotype abnormalities and molecular mutations,[170,172,173] indicating that LSCs have common intrinsic characteristics. Resistance to BCL2 inhibition was observed in AMLs with high expression of the anti-apoptotic protein MCL-1, but these AMLs were shown to be targeted by combined treatment with BCL2 and MCL-1 inhibitors.[174–177]

Paradoxically, certain leukemia-associated mutations can reinforce an increased dependency of LSCs on anti-apoptotic proteins. For instance, IDH1/2 mutants were shown to directly inhibit complex IV activity, thereby lowering the mitochondrial activity and increasing the dependency on apoptosis inhibitors such as BCL2 in order to prevent induction of cell death pathways triggered by a very low mitochondrial membrane

potential.[172] A recent study aimed to identify pathways whose interference might confer increased sensitivity of AML cells to BCL2 inhibition and identified several metabolic pathways including OXPHOS, nucleotide biosynthesis and the heme biosynthesis pathway.[156] Mechanistically, heme depletion triggered impaired function of the mitochondrial ETC and caused lowering of mitochondrial membrane potential, thereby potentiating Venetoclax-induced apoptosis. This finding supports the concept that cells with low mitochondrial activity, such as LSCs, have an increased dependency on anti-apoptotic pathways. Clinical trials have shown promising results for treatment of relapsed/refractory AML when Venetoclax was used as monotherapy,[178] as well as for newly-diagnosed, elderly AML patients when Venetoclax was used in combination with hypomethylating agents.[179] However, Venetoclax-based treatment seems to work less efficiently in relapsed patients compared to previously untreated patients.[180] Apparently, relapsed LSCs acquire different metabolic properties and fuel OXPHOS via different routes, for instance by upregulation of fatty acid metabolism.[5] Therefore, they seem to be less dependent on BCL2 for maintaining sufficiently high mitochondrial activity to ensure their survival.

8.2 Targeting autophagy to eradicate LSCs

Autophagy is an important strategy for avoiding accumulation of damaged proteins and organelles that eventually can lead to cell death. For leukemia cells, functional autophagy was found to be crucial for leukemic transformation and for impairing cellular response to chemotherapy (reviewed in [181]). High expression of genes involved in autophagy regulation was shown to be associated with poor prognosis in AML,[63] and several recent studies have suggested autophagy inhibition as a potential strategy for targeting LSCs.[4,52,131] ROS-low CD34$^+$ AMLs were shown to have higher basal levels of autophagy and increased sensitivity to autophagy compared to their ROS-high CD34$^+$ counterparts.[52] Pei et al. identified factors that are expressed differently in ROS-low AML LSCs compared to the ROS-high non-LSC fraction and reported a crucial role of the mitophagy-related protein FIS1 (mitochondrial fission protein 1),[4] which highlights the importance of mitochondrial dynamics for LSC maintenance (**Figure 3B**). Knockdown of FIS1 was shown to severely impair the stem and progenitor potential of AML LSCs, but not of normal HSCs,[4] and AML patients with a high FIS1 expression were less likely to respond to chemotherapy.[182] Similarly, increased activation of the fission protein DRP1 in T-ALL cells by bone marrow-niche mediated stimuli was shown to lower ROS levels, change the mitochondrial phenotype to a more fragmented morphology and improve chemoresistance.[47] Therefore, increased levels of autophagy seem to contribute significantly to the characteristic features of LSCs, and targeting autophagy can impair LSC survival.

9 Do ROS-low LCS drive AML relapse after chemotherapy?

Chemotherapy of AML patients frequently includes treatment with the pyrimidine nucleoside analog cytarabine in combination with anthracyclines.

While this strategy can lead to an initial reduction of leukemic blasts in the majority of patients, the 5-year overall survival rate of patients <60 years has not significantly improved in recent decades and ranges from 35% to 40%.[183] Adverse outcomes of AML are mainly associated with leukemia relapse, which is assumed to be driven by LSCs that are not targeted by chemotherapy and retain their disease-initiating properties.[89] This model is supported by the increasing knowledge about characteristic ROS-low LSC properties, since features such as low metabolic activity, increased BCL2 expression and high levels of autophagy can confer increased chemotherapy resistance as described above. Additionally, our group recently observed increased expression of the drug efflux transporter ABCB1 in the ROS-low defined LSC compartment (unpublished results), and high expression of ABC transporters in leukemia cells has been linked to poor treatment outcome in AML patients.[184,185] Moreover, several longitudinal sequencing studies have strongly supported the concept that relapse is driven by a resistant cell population that is already present prior to chemotherapy treatment, and is not generated as a consequence of the mutagenic properties of the chemotherapeutic drugs.[89,186–188] However, it has not been shown that the ROS-low LSC population drives disease relapse. The promising results of clinical trials in which standard treatments were combined with Venetoclax are thought to be a result of efficient targeting of the ROS-low LSC population,[189] and therefore support the hypothesis that ROS-low LSCs drive disease recurrence after conventional chemotherapy. However, other studies reported that chemoresistant cells have features distinct from those of ROS-low cells: Farge et al. showed that the AML cells that persist after chemotherapy treatment using cytarabine for 5 days have increased mitochondrial mass, mitochondrial membrane potential and ROS production – and thus have features somewhat opposite to ROS-low LSCs.[60] Furthermore, other studies reported that treatment with cytarabine does not enrich for functional LSCs[190] and efficiently targets the $CD34^+CD38^-$ cell population.[191] Notably, our group recently observed that ROS-low LSCs are enriched for $CD34^+CD38^-$ cells (unpublished results), indicating that metabolic LSC characteristics do overlap with previously described phenotypic LSC features such as CD34/CD38 expression.[192] These conflicting reports on the characteristics of chemoresistant cells can likely be explained by differences between steady-state LSCs and LSCs that were exposed to chemotherapy. LSCs have been shown to undergo phenotypic changes during the course of chemotherapy,[193] and this might also apply to their metabolic characteristics. Consequently, LSCs with low ROS levels could be indeed the cell population that is most resistant to chemotherapeutic drugs, but they might respond to the severe therapy-related stress with upregulation (transient or otherwise) of their mitochondrial activity.

10 Conclusion

Cellular ROS levels inversely correlate with the functionality of both normal stem cells and leukemic stem cells. Low ROS levels apparently reflect a metabolic state of relatively low mitochondrial activity, which coincides with the low

mitochondrial membrane potential, low levels of cellular ATP and the small, fragmented mitochondria morphology found in both HSCs and LSCs. Avoiding accumulation of ROS and maintaining mitochondrial stability is essential for both types of stem cells in order to prevent ROS-mediated cell damage and induction of cell death pathways mediated by mitochondrial dysfunction. Multiple gene knockout studies have demonstrated that targeting metabolic pathways such as glycolysis and oxidative phosphorylation, or redox-regulating machineries such as autophagy and anti-oxidative enzymes, interferes with stem cell maintenance. In steady-state, however, HSCs and LSCs seem to rely on different strategies to maintain their characteristic ROS-low condition. HSCs reside in a hypoxic bone marrow niche and preferentially use glycolysis for their energy generation, which results in reduced ROS production compared to ATP production by mitochondrial oxidative phosphorylation. In contrast, LSCs rely strongly on their remaining mitochondrial activity, which they maintain through mitophagy and mechanisms dependent on the anti-apoptotic protein and master regulator of mitochondrial activity BCL2. The strong dependency of LSCs on BCL2 and certain key players in mitophagy seems to be a characteristic feature of LSCs, and is not observed to this extent in normal HSCs or in the total mononuclear AML cell population. Therefore, interference with those pathways is a promising strategy for eradicating LSCs, which are apparently not efficiently targeted by standard chemotherapy approaches.

An interesting question that has not been completely answered is whether the higher ROS levels observed in the total leukemia cell population compared to ROS-low LSCs have a causative role in reducing stemness potential, or if they are simply a reflection of a different metabolic state that is not compatible with stem cell function. A recent study showed that standard induction chemotherapy leads to elevation of ROS, but does not efficiently target LSCs, indicating that increased ROS levels alone are not sufficient to compromise LSC viability or function.[7] To improve current therapy protocols, future studies should continue to investigate the metabolic properties of LSCs and shed light on this question.

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